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Morphogen-Based Chemical Flypaper for *Agaricia humilis* Coral Larvae

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Abstract. Larvae of the scleractinian coral *Agaricia humilis* settle and metamorphose in response to chemosensory recognition of a morphogen on the surfaces of *Hydrolithon boergesenii* and certain other crustose coralline red algae. The requirement of the larva for this inducer apparently helps to determine the spatial pattern of recruitment in the natural environment. Previous research showed that the inducer is associated with the insoluble cell wall fraction of the recruiting algae or their microbial epibionts, and that a soluble but unstable fragment of the inducing molecule can be liberated by limited hydrolysis, either with alkali or with enzymes specific for cell wall polysaccharides. We now show that the parent morphogen can be solubilized by gentle decalcification of the algal cell walls with the chelators EGTA or EDTA, suggesting that the morphogen may be a component of the calcified recruiting alga itself, rather than a product of any non-calcified microbial epibionts. The solubilized inducer is subsequently purified by hydrophobic-interaction and DEAE chromatography. The purified, amphipathic morphogen retains activity when tightly bound to beads of a hydrophobic-interaction chromatography resin, and this activity (tested with laboratory-reared larvae) is identical in the ocean and the laboratory. We have attached the purified, resin-bound inducer to surfaces coated with a silicone adhesive and thus produced a potent artificial recruiting substratum—*i.e.*, a morphogen-based chemical "flypaper" for *A. humilis* larvae. This material should prove useful in resolving the role of chemosensory recognition of morphogens in the control of substratum-specific settlement, metamorphosis, and recruitment and in the maintenance of species isolation mechanisms in the natural environment.

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Abbreviations: DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol-bis(β-amino ethyl ether) N,N,N¹,N¹-tetraacetic acid; HIC, hydrophobic-interaction chromatography; Tris, tris-hydroxymethylaminomethane.

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Introduction

Chemosensory recognition of unique substrata (algae, microbial films, conspecifics, *etc.*) has been found sufficient, in the laboratory, to induce settlement and metamorphosis of the larvae of a wide variety of marine invertebrate taxa (for reviews see Crisp, 1974, 1984; Scheltema, 1974; Hadfield, 1978, 1986; Chia, 1978; Burke, 1983, 1986; Morse, 1985, 1990; Rittschof and Bonaventura, 1986; Hadfield and Pennington, 1990; Pawlik, 1990; Morse, 1992). In a number of cases, the specificity of this response demonstrated in the laboratory parallels the substratum-specificity of settlement, metamorphosis, and recruitment in the natural environment, and the complex inducer (or a partially purified fraction or presumptive chemical analog) induces substratum-specific settlement and metamorphosis of the target species in the ocean (Crisp, 1974; Morse *et al.*, 1980, 1988; Highsmith, 1982; Sebens, 1983; Hadfield and Scheuer, 1985; Connell, 1985; Raimondi, 1988, 1990a, b; Jensen and Morse, 1984, 1990; Morse and Morse, 1984; Shepherd and Turner, 1985; Raimondi and Schmitt, 1992). Using artificial substrata with or without a sticky coating, Walters (1992) showed that rugophilic barnacle and bryozoan larvae actively select microhabitats for metamorphosis that differ from the sites of their first contact with the substrate. Not yet clear, however, is the extent to which larval recognition

of any *individual, natural morphogenic molecule* may contribute to the control of recruitment and to species isolation in the natural environment, and how this recognition interacts with other determining factors (such as surface rugosity, hydrodynamic regime, other biological interactions, *etc.*) that also are usually present with the chemical cue in the complex natural substratum (*cf.* Butman, 1987; Pawlik *et al.*, 1991). These effects must be resolved if we are to understand the role of larval chemosensory receptors in the control of recruitment and their possible contribution to the evolution of species isolation. What is needed for such experimental resolution (in those cases in which the natural cue is normally nondiffusible) is a kind of morphogen-based chemical "flypaper," an inert substratum to which the highly purified natural morphogen is bound, permitting dissection of the contributions and interactions of the presence of the individual chemical cue and other features of the recruiting substratum and the environment.

As we show here, properties of the alga-associated morphogen of the common Caribbean scleractinian coral *Agaricia humilis* facilitate both its purification and the development of an immobilized, morphogen-based recruiting substratum. Larvae of *A. humilis* are induced to settle and metamorphose by contact-dependent chemosensory recognition of molecules on the surfaces of *Hydrolithon boergesenii* and other specific crustose coralline red algae (Morse *et al.*, 1988; Morse and Morse, 1991). In the absence of this cue, larvae persist without settlement or metamorphosis for ≥30 days in the laboratory (Morse and Morse, 1991). This larval requirement for a substratum-specific morphogen parallels, and thus presumably helps shape, the pattern of recruitment of this species on coralline red algae in the natural environment (Morse *et al.*, 1988).

We previously found that the nondiffusible inducer of settlement and metamorphosis is associated with the insoluble cell wall fraction of the recruiting alga or its microbial epibionts (Morse *et al.*, 1988; Morse and Morse, 1991). We also found that a small (<2000 Da), soluble fragment of the inducing molecule could be liberated by decalcification of the algal cell walls with dilute acetic acid, followed by limited hydrolysis with alkali or with purified enzymes that hydrolyze specific bonds found in cell wall polymers (Morse and Morse, 1991). This biochemical result was consistent with either an algal or a bacterial origin of the inducer, as similar polymers can be found in the cell walls of both groups. Furthermore, the solubilized morphogen fragment was obtained in low yield and proved to be extremely unstable, hindering both its further purification and use.

We now show that the morphogen can be solubilized without hydrolysis, simply by gentle decalcification of the algal cell walls (or other divalent metal-cross-linked insoluble phycocolloids) with the calcium chelator EGTA, or with the divalent metal chelator EDTA, used at the pH of seawater. The fact that the release of the inducer in soluble form is dependent on decalcification strongly suggests that it may be a component of the calcified alga itself, rather than a molecule produced by any associated (non-calcified) microbial epibionts. Significant purification and concentration of the solubilized, amphipathic molecule are achieved by adsorption to a hydrophobic chromatographic support that strongly binds the inducer from high salt, and subsequently releases the molecule at low salt concentration. Further purification then is obtained by anion-exchange chromatography on DEAE. Our data suggest that the morphogen extracted and purified by these procedures has an apparent (or micellar) molecular weight of 6000–8000 Da, and is the parent molecule from which the smaller, unstable hydrolysis fragment originally had been obtained.

The properties of this molecule make it suitable for tests of the role of chemical induction of larval settlement and metamorphosis, both in the laboratory and the ocean environment. The purified morphogen retains activity when adsorbed to beads of the hydrophobic interaction chromatography resin, and it remains bound to the resin in seawater. Activity of the resin-bound inducer is identical in the ocean and the laboratory when tested with *A. humilis* larvae produced in the laboratory. Attachment of the resin-bound inducer to surfaces coated with a silicone adhesive produces a potent artificial recruiting substratum corresponding to a morphogen-based chemical flypaper for *A. humilis* larvae.

Materials and Methods

Larvae

Coral larvae were produced and maintained in culture as described previously (Morse *et al.*, 1988; Morse and Morse, 1991). Adult colonies of *Agaricia humilis*, identified by the taxonomic criteria of Wells (1973) and van Moorsel (1983, 1989), were collected from depths of 1–5 m in Bonaire, Netherlands Antilles, and incubated in the dark in 20–60 l of seawater at 28°C. Planula larvae were released copiously between 1800 and 2300 h, collected from the surface with a Pasteur pipet, and maintained at densities of ≤0.5 larva/ml in 600-ml polyethylene containers of 0.2-μm-filtered seawater containing 2 μg/ml of the antibacterial antibiotic rifampicin at 28°C, under continuous indirect illumination from a 40-W incandescent bulb at 3 m.

Purification of the morphogen

All operations were at 24–28°C unless otherwise noted. *Hydrolithon boergesenii* was cleaned of macroscopic epi-

bionts, scraped from its substratum, blotted dry, and extracted fresh or stored frozen until use. The fresh or frozen coralline alga was ground (in 1-g portions) with a mortar and pestle until a smooth paste was obtained; it was then ground further after the addition of 10 ml of 0.2-μm-filtered seawater containing 2 μg/ml rifampicin (RFSW)/g alga. The algal homogenate was diluted to 40 ml with RFSW, centrifuged 5 min at 10,000 $\times$ *g*, and the aqueous supernatant discarded. The water-insoluble pellet was resuspended in RFSW and washed by centrifugation two more times, as above, and then (except as specified in Table I) added to a solution (1 l/g alga) of 50 m*M* EDTA $(Na)_3$ that had first been adjusted to pH 8.2. Alternatively, EGTA adjusted to pH 8.2 with NaOH was used. Decalcification was allowed to proceed by magnetic stirring of the resulting suspension for 22–24 h, after which the decalcified soluble extract was readjusted to pH 8.2 if necessary, and clarified by vacuum filtration through glass fiber filters (Millipore GFC filters, 47 mm diam., using 1 filter/250 ml).

The resulting clarified demineralized crude extract was adjusted to 2 *M* NaCl to facilitate adsorption of the morphogen to beads of a t-butyl hydrophobic interaction chromatography (HIC) resin (Biorad, Richmond, CA). After adding 2 g (wet weight) of the water-washed and blotted HIC resin/liter, the resin was stirred with the crude inducer for 3 h, and the resin then collected by vacuum filtration on a GFC (47 mm) filter. The filtrate was refiltered through the resin filter-cake two more times, and the resin rinsed on the filter with two 10-ml portions of 2 *M* NaCl to remove traces of the chelator. The resin then was blotted with paper towels to remove excess salt, resuspended in a minimal volume of pre-chilled Tris Cl buffer (1 m*M*, pH 8.2, at 2°C; 3 ml/l of original clarified, demineralized crude extract), and poured into a chromatography column (0.9 cm diam.). The column was eluted with the same chilled buffer and the aid of a low-pressure chromatography pump; fractions (3 ml/l of original clarified, demineralized crude extract) were collected and assayed for morphogenic activity, absorbance, and protein. Fractions containing the peak of activity were pooled. In a typical preparation, 4 $\times$ 1 g portions of *Hydrolithon boergesenii* were individually homogenized, pooled, and decalcified with 4 l of chelator; the resulting solubilized inducer was then adsorbed to 8 g of HIC resin, eluted in 6–10 fractions of 12-ml each, and 3–4 fractions containing the peak of activity were pooled.

Further purification of the morphogen was achieved by anion-exchange chromatography over Trisacryl-DEAE (from LKB) or DEAE-Sephadex (from Pharmacia). A sample of the pooled, most active fractions of the HIC eluate was diluted with an equal volume of Tris Cl buffer (1 m*M*, pH 8.2), and adsorbed to DEAE-resin in a chromatography column (2-ml bed volume/10-ml applied sample; 0.9-cm column diam.). The active morphogen then was eluted with a gradient of increasing NaCl concentration (from 0 to 0.6 *M*) in Tris Cl buffer (1 m*M*, pH 8.2).

Assays of the morphogen in the laboratory

Assays for the induction of larval attachment and metamorphosis (unless otherwise noted) were performed in duplicate samples of 5 larvae/sample, in 10 ml of seawater (0.22-μm-filtered) contained in 20-ml polystyrene disposable breakers incubated at 28°C with indirect illumination (as described above) for 18–24 h; results shown are the average percent metamorphosis $\pm$ deviation from the mean. Metamorphosis, defined as the differentiation and calcification of the septa following permanent attachment and cellular differentiation, was quantified by examination with a binocular microscope. This transition is an unequivocally recognized (and in this species, irreversible) developmental event, and not simply a behavioral change (Morse *et al.*, 1988).

The extracted morphogen was assayed either by direct addition in solution or after adsorption to a hydrophobic support. Following elution from the HIC or DEAE chromatographic resins, samples of the concentrated inducer were assayed directly in solution; up to 1.5 ml of concentrated inducer in 1 m*M* Tris Cl buffer (with 0–0.6 *M* NaCl) could be assayed by direct addition to the larvae in 10 ml filtered seawater. Dilute samples of the inducer in the clarified, demineralized crude extract were assayed by prior adsorption from 2 *M* NaCl to the HIC resin (as described above) or to hydrophobic nitrocellulose filters; the resin beads or the minced filters then were added directly to the small beakers of seawater, and the larvae added for assay. Samples of the chromatographically purified inducer also were assayed after readsorption to the HIC resin. Modifications of the standard assay procedure are described below.

Characterization of the morphogen

The molecular weight of the HIC-purified morphogen was estimated by gel-filtration chromatography (Bio-Gel P6 acrylamide resin; from Biorad), and by dialysis and ultrafiltration (Amicon) using calibrated porosity membranes. Methods were standard, as described previously (Morse and Morse, 1991). Treatment of the HIC-purified morphogen with trypsin was performed with the enzyme covalently linked to insoluble beads (from Sigma, St Louis, MO) to facilitate removal of the protease prior to assays of the remaining morphogenic activity. Aliquots of the purified morphogen (2 ml) were incubated with 0.2 g immobilized trypsin (187 units/g polyacrylamide beads) with

gentle magnetic stirring for 5 h at 28°C, and then separated from the immobilized enzyme by passage (with air pressure) through a small chromatography column that retained the insoluble beads. Parallel incubations were conducted with samples of buffer stirred with equal amounts of the enzyme-linked beads; the treated buffer was separated from the enzyme and subsequently combined with the stirred but untreated morphogen prior to assay. These controls demonstrated that any leaching of the trypsin from its solid support was minimal, as were direct effects on the larvae. Protein concentrations were estimated by the method of Bradford (1976). Adsorbance was measured with a Beckman DU-7 scanning spectrophotometer.

Assays of the morphogen in the field

Because previous laboratory-based studies of settlement have in many cases yielded results not directly reproducible in the natural environment, they have been of little value in predicting the behavior and understanding the ecology of the species under investigation. To overcome this obstacle it was necessary to develop a method for redeploying the purified natural morphogen for tests in the ocean, to determine whether the molecule we had purified retained its activity in the natural environment. The experiment described below allowed us to compare the activity of the chromatographically purified morphogen under laboratory and ocean conditions, the first essential step toward the development of a morphogen-based larval flypaper.

We took advantage of the hydrophobic property of the morphogen to develop a material useful for testing its activity in the ocean environment. After chromatographic purification, the molecule was readsorbed to fresh beads of HIC resin from 2 *M* NaCl, as described above. This resin-bound morphogen retained full activity after washing with several liters of seawater, making it possible to test the efficacy of the HIC-resin-bound morphogen directly in the ocean. To compare the activities of the HIC-resin-bound morphogen in the ocean and the laboratory, minor modifications in the standard assay procedure were employed. Assays were conducted in clear plastic vials (25 × 50 mm, i.d. × h) with the caps replaced by tightly secured mesh (Nitex; 100 μm) sufficient to retain *A. humilis* larvae but permit exchange with environmental seawater (*cf.* Raimondi and Schmitt, 1992, for similar technique). HIC resin (with or without adsorbed morphogen) and 10 laboratory-reared larvae were added to each vial containing fresh seawater, and identical sets were incubated simultaneously in the laboratory and in the ocean. Incubations in the ocean were accomplished by securing the vials with cable-ties to an anchored line maintained in a vertical orientation with a subsurface buoy. The ocean tests were conducted at a depth of 7 m, in the habitat from which the parental *A. humilis* colonies had been obtained. The vials were recovered and examined microscopically for metamorphosis after 22 h.

Incorporation of the purified morphogen on synthetic surfaces

A sample of HIC-purified morphogen (22 ml) was adjusted to 2 *M* NaCl and readsorbed to 0.5 g fresh t-butyl HIC resin as described above; 30 mg of the resulting morphogenically active resin cemented to the bottoms of three polystyrene beakers (3.2-cm diam.; 50-ml capacity) with Dow-Corning silicone adhesive. The beakers were immediately filled with 0.22-μm-filtered seawater, and the unadhered resin and byproducts of adhesive curing were removed by four successive replacements of this seawater over 1 h. After this curing, the silicone resin was no longer sticky. An estimated 50% of the added resin (*ca.* 15 ± 5 mg/beaker = *ca.* 2 mg resin/cm^2) was estimated to remain firmly cemented to the polystyrene surfaces. Water-washed HIC resin without adsorbed morphogen was used to prepare the control surfaces.

Results

Purification of the morphogen

We found that gentle decalcification with either of the chelators, EGTA or EDTA, is sufficient to solubilize significant morphogenic activity from the water-insoluble fraction of the coralline alga *Hydrolithon boergesenii*. The treatment employed completely decalcified the algal cell walls. The properties and subsequent purification behavior of the morphogen solubilized with the two different chelators are thus far indistinguishable; because EDTA is less expensive than EGTA, and because it exhibits superior buffering capacity, we chose to use EDTA routinely. The fact that the inducer is solubilized by the chelators at pH 8.2 (the ambient pH of seawater at the shallow collection sites in Bonaire) suggests that solubilization is achieved without appreciable hydrolysis. The chelator-solubilized morphogen is markedly amphipathic—*i.e.*, it exhibits both hydrophobic and hydrophilic properties. It is this property that has been exploited to concentrate and purify the morphogen and to couple it to an inert support, in which form it can act as a kind of morphogenic flypaper for *A. humilis* larvae.

Because of the amphipathic behavior of the solubilized morphogen, it can be adsorbed from the dilute, decalcified, and clarified crude extract (see Materials and Methods) and concentrated on hydrophobic supports. The addition of NaCl to the clarified crude extract greatly enhances this binding to nitrocellulose filters and to beads of the t-butyl

hydrophobic interaction chromatographic resin. The morphogenic inducer retains its activity after adsorption to these hydrophobic supports and is not eluted by seawater (although penetrance into the pores of the nitrocellulose filters apparently reduces detectability by the larvae, requiring that the filters be minced before assay). The adsorbed inducer can be subsequently released from the HIC support by elution with low ionic strength buffer (Fig. 1). This chromatographic adsorption and subsequent elution concentrated the active morphogen by about 100-fold from the dilute demineralized crude extract (permitting direct assays of the morphogen in solution), resulting in significant purification. As seen in Figure 1, the peak of morphogenic activity is eluted in fractions 2–5, whereas the majority of the light-absorbing contaminants are eluted in the first fraction. At the lower concentrations assayed, activity of the purified inducer is approximately proportional to the amount added (Fig. 2). Induction of metamorphosis is both time- and dose-dependent, with metamorphosis observed as quickly as 2–3 h following addition of high concentrations of the HIC-purified inducer.

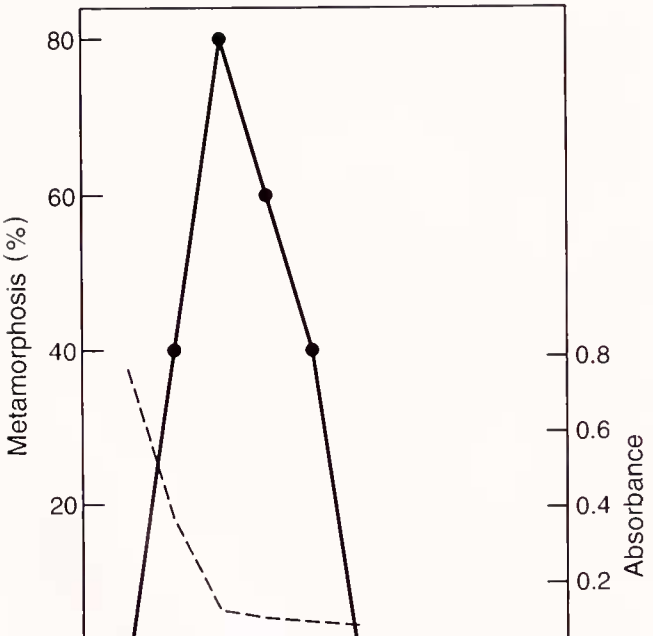

Figure 1. Hydrophobic-interaction chromatography. The clarified demineralized filtrate (4 l) was adjusted to 2 *M* NaCl, adsorbed to 8 g of the t-butyl HIC resin, poured into a column, and eluted with Tris Cl buffer as described in Materials and Methods. Fractions of 12 ml were collected; 0.4 ml of each fraction was assayed for morphogenic activity (solid line). Absorbance at 212 nm is shown (dashed line).

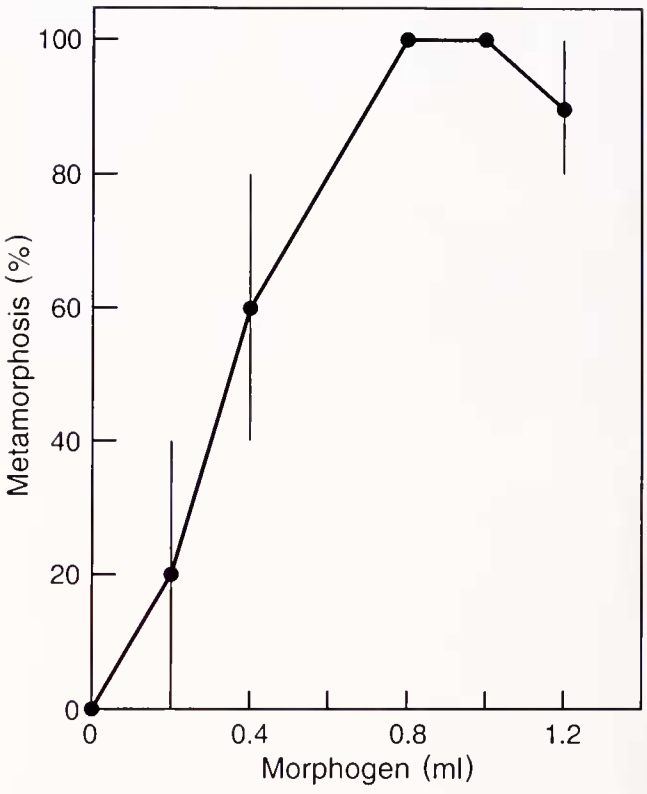

Figure 2. Activity of the HIC-purified and concentrated morphogen. Samples (0.2–1.2 ml) of the pooled morphogen eluted in the peak fractions from the HIC resin (fractions 2–5 from Fig. 1) were assayed in duplicate for morphogenic activity. Controls = 0.5 ml Tris Cl buffer with no morphogen. Error bars indicate deviation from the mean.

The yield of morphogen that can be solubilized (and subsequently purified by HIC) is strongly dependent on the concentration of chelator in the initial extract (Table 1). The results shown in Table IA demonstrate that relatively little inducer is solubilized by prolonged (20 h) stirring of the water-washed crude cell wall fraction in aqueous buffer in the absence of chelator, and that solubilization increases markedly when chelator (0.05 *M* EDTA) is present. The absolute *concentration* of the chelator also is critical to obtain high yields of the soluble morphogen. Thus (Table IB), in three parallel extractions using the same total *amount* of chelator (50 mmole EDTA/g alga), sufficient to completely decalcify the cell walls in all three samples, progressively lower yields of soluble inducer are obtained with progressively higher *concentrations* of EDTA. This behavior is consistent with the hydrophobic property of the amphipathic morphogen; at progressively higher concentrations of the highly charged EDTA salt [nominally, $(Na^+)_4\ EDTA^{-4}$], the hydrophobic behavior of the morphogen becomes increasingly dominant, apparently causing its retention by adsorption to other hydrophobic molecules (possibly, for

example, the chlorophyll and phaeopigments known to be present) in the water-insoluble algal residue.

The morphogen eluted from the HIC resin can be further purified by anion-exchange chromatography (Fig. 3). It binds ionically to DEAE at low salt concentration and is eluted by a gradient of increasing NaCl molarity. The activity elutes from a Trisacryl-DEAE column with a symmetrical peak in the range of 0.3–0.5 *M* NaCl, indicating moderately strong anionic binding. Similar results (not shown) were obtained using a DEAE-Sephadex chromatographic resin, although the resolution obtained with the Trisacryl (acrylamide-based) ion-exchanger was superior to that obtained with the polysaccharide-based Sephadex resin. From the ratios of morphogenic activity to absorbances, we estimate that the inducer has been purified ≥1000-fold (total purification, relative to the starting crude algal homogenate) after the hydrophobic-interaction chromatography, and ≥4000-fold after the anion-exchange chromatography.

Characterization of the morphogen

The apparent molecular weight of the morphogen eluted from the HIC resin was estimated by analyzing the distribution of morphogenic activity after gel-filtration over Bio-Gel P6, and after dialysis and ultrafiltration through a series of calibrated-porosity membranes. These results yielded estimates consistent with a weight-average apparent molecular weight in the range of 6000–8000 Da; however, this value may be influenced by the ability of the purified amphipathic morphogen to form micellar aggregates. The DEAE-purified morphogen is negative in tests for protein by the Bradford (1976) procedure, although it displays an absorption maximum in the ultraviolet at *ca.* 210–212 nm. The apparent insensitivity of morphogenic activity to trypsin (Table II) indicates that if any peptide is present, it does not contain lysine or arginine in a region essential for activity. The chelator-solubilized and chromatographically purified morphogen retains activity for ≥24 h at 28°C, and for several weeks when frozen.

The properties of this molecule thus far characterized suggest that the chelator-solubilized and chromatographically purified morphogen may be the parent molecule from which the smaller fragment had previously been obtained by enzymatic or alkaline hydrolysis, following demineralization with acetic acid (Table III). The chelator-solubilized morphogen is apparently larger and is more stable than the hydrolytic fragment. The chelator-solubilized inducer contains both hydrophobic and anionic domains, whereas the fragment apparently lacks the hydrophobic domain.

Table I

Solubilization of the morphogen depends on chelator concentration

Experiment	EDTA (mmoles/g alga)	[EDTA] (*M*)	Metamorphosis (%)
A	0	0	10 ± 10
	50	.05	90 ± 10
B	50	.05	90 ± 10
	50	.20	30 ± 30
	50	.50	0 ± 0

Experiment A: Equal portions (2 g each) of a washed, water-insoluble algal homogenate were extracted with 2 l (Na) EDTA (0.05 *M*) or Tris Cl buffer (5 m*M*) by prolonged stirring (20 h; 28°C; pH 8.2). The extracts were clarified, and morphogenic activity in the soluble phase was determined after HIC adsorption and elution as described in Materials and Methods (0.8 ml/assay).

Experiment B: Equal portions (0.3 g each) of a washed, water-insoluble algal homogenate were extracted (20 h; 20°C; pH 8.2) with (Na) EDTA solutions, all containing 15 mmoles of EDTA at the indicated concentrations (*i.e.*, in 30, 75, or 300 ml). The extracts were clarified, and the morphogenic activity in the soluble phase from each was determined by the nitrocellulose-binding assay, as described in Materials and Methods.

Activity of the morphogen in the field

We found that the hydrophobic property of the morphogen can be exploited not only for its purification, but also to develop a material useful for testing its activity in the ocean. Adsorption of the chelator-solubilized morphogen to the HIC resin is reversibly controlled by the ambient salt concentration. After chromatographic purification, the molecule was re-adsorbed to fresh beads of HIC resin from high salt (as described in Materials and Methods). The activity and other properties of the HIC-resin-bound morphogen prepared in this manner proved indistinguishable from those of the resin-bound morphogen prepared directly from the clarified chelator extract. Furthermore, the resin-bound morphogen retained full activity after extensive washing with seawater (*ca.* 0.4 *M* in NaCl), indicating that it would be possible to compare the activities of the HIC resin-bound morphogen in the ocean and the laboratory.

The results of this comparison (Fig. 4) demonstrate that the activity of the HIC-resin-bound morphogen is identical in the ocean and the laboratory—and that relatively small quantities of the morphogen-containing resin are sufficient to induce attachment and metamorphosis of the larvae. The results were similar whether the resin-bound morphogen was prepared from chromatographically purified morphogen or from the clarified chelator solution. In contrast, the control HIC resin, treated in parallel but with no adsorbed morphogen, was inactive. The HIC-resin-bound morphogen loses activity after 1–2 days at 28°C, but remains stable for several weeks at 2°C; this

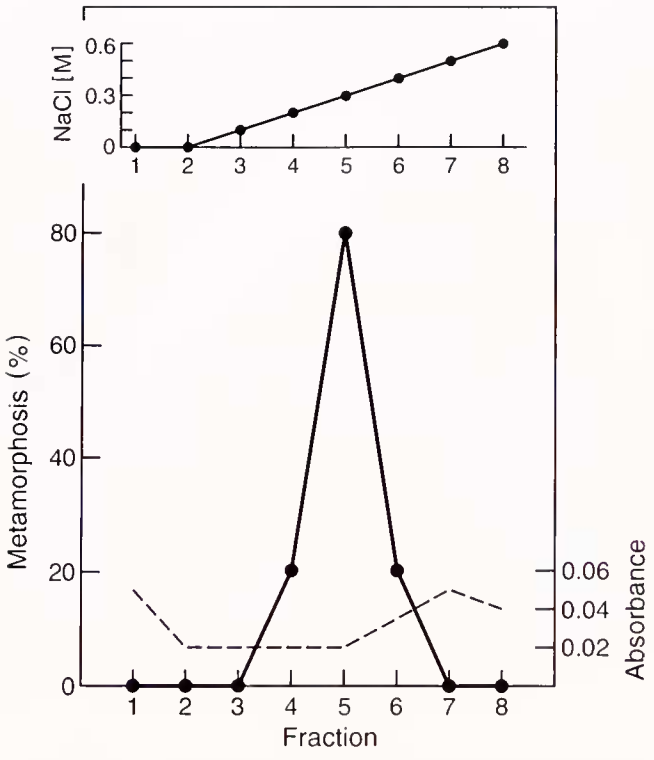

Figure 3. Anion-exchange chromatography. Five ml of the pooled morphogen eluted in the peak fractions from the HIC resin (fractions 2–5 from Fig. 1) was diluted and chromatographed on Trisacryl-DEAE. Elution from the DEAE was performed with a gradient of increasing NaCl concentration (inset) as described in Materials and Methods. Fractions of 5 ml were collected; 1.5 ml of each fraction was assayed for morphogenic activity (solid line). Absorbance at 212 nm is shown (dashed line); the bulk of the applied absorbance remained bound on the column.

behavior is comparable to that of the purified inducer in solution.

Morphogen-based larval flypaper

To produce a practical experimental tool that could be deployed in the natural ocean environment, we incorporated the resin-bound morphogen on a simple, stable platform. We found that the HIC-resin-bound morphogen (prepared from the chromatographically purified morphogen) can be attached to surfaces with a waterproof silicone adhesive, without significant loss of activity. The

Table II

The purified morphogen is insensitive to trypsin

Trypsin treatment	Metamorphosis (%)
Control	90 ± 10
Treated	80 ± 10

HIC-purified morphogen was incubated with immobilized trypsin, separated from the enzyme, and assayed for remaining activity as described in Materials and Methods. Control was a sample of the untreated morphogen, incubated in parallel, and then combined with a sample of buffer that had been incubated in parallel with the immobilized enzyme.

Table III

Chelator solubilizes the parent morphogen

	Morphogen Solubilized By	
Property	Chelator[1]	Enzymes or alkali[2]
M (Da)[3]	6000–8000	≤2000
Stability	Higher	Low
Binding		
Hydrophobic	Yes	No
Anionic	Yes	Yes

[1] This study.
[2] Data from Morse and Morse, 1991.
[3] Apparent molecular weight.

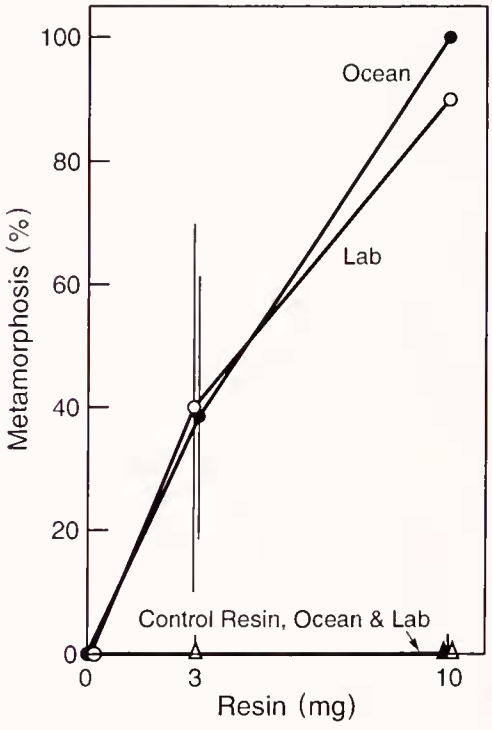

Figure 4. Activity of the HIC resin-bound morphogen in the ocean and the laboratory. Samples of the t-butyl HIC resin, either with adsorbed morphogen (circles) or without morphogen (triangles), were prepared and assayed in mesh-capped vials, in the ocean (filled symbols) and the laboratory (open symbols), as described in the text. Samples were assayed with 10 larvae/vial; results shown are the means ± S.E. (where no error bars are shown, S.E. = 0). Values for 3 mg are displaced horizontally for clarity.

result is a potent morphogen-based larval flypaper that is recognized efficiently by *A. humilis* larvae when tested in the laboratory (Table IV). Larvae exposed to this morphogen-bearing surface rapidly began attachment and metamorphosis, and all of the metamorphosed individuals were found attached to the morphogen-containing surfaces. In contrast, exposures to the comparable surfaces of attached resin that lacked the purified morphogen resulted in no settlement or metamorphosis; the larvae in these treatments continued to swim normally. In a parallel experiment, when the morphogen-based flypaper was prepared either with or without grooves (*ca.* 1-mm deep and 1-mm wide), all of the larvae that settled and metamorphosed in the presence of the grooved substratum did so within the grooves (in contact with the resin-bound morphogen), although the numbers of larvae that settled and metamorphosed were identical on the grooved and smooth substrata. Post-metamorphic growth through development of the feeding polyps occurred normally on the artificial substrata, with no inhibition relative to that seen on the natural host coralline alga. This material should thus make it possible to assess the relative contributions of chemosensory recognition and other factors (texture, hydrodynamics, light, biological interactions, *etc.*) to the control of substratum specificity, settlement, metamorphosis, and recruitment both in the laboratory and, with natural propagules, in the ocean.

Table IV

Morphogen-based chemical flypaper for Agaricia humilis *larvae*

Morphogen on surface	Metamorphosed/total
	9/10
+	9/10
	10/10
	0/10
−	0/10
	0/10

t-Butyl HIC resin, either with adsorbed chromatographically purified morphogen, or without, was cemented to polystyrene surfaces which were then extensively washed with seawater as described in Methods. The prepared surfaces contained *ca.* 2 mg resin/cm^2 and 8 cm^2/trial; each was assayed for morphogenic activity with 10 larvae in 40 ml filtered seawater. Attachment and metamorphosis were observed as early as 2 h, and scored after 21 h; all metamorphoses occurred on the resin-coated surfaces.

Discussion

The natural morphogen recognized by *Agaricia humilis* larvae has been purified ≥4000-fold in three successive steps: differential solubilization (in which the water-soluble molecules are first extracted and discarded, and then the chelator-solubilized molecules are subsequently released); hydrophobic-interaction chromatography; and anion-exchange chromatography. The first step separates the morphogen from the bulk of the algal phycocolloids, proteins, and small molecules. HIC removes the majority of hydrophilic contaminants, and the final DEAE chromatography resolves the inducer both from species more weakly and more strongly anionic than the morphogen. Structural studies of the purified morphogen are now in progress.

The properties of the amphipathic morphogen solubilized from the source alga by gentle demineralization (at the pH of seawater) with the calcium-specific chelator EGTA or its analog, the divalent metal chelator EDTA, suggest that this may be the parent molecule from which the smaller and less stable fragment originally had been obtained by limited enzymatic or alkaline hydrolysis following cell wall decalcification with acetic acid. The fact that solubilization is strongly dependent on demineralization suggests that the inducer of *A. humilis* settlement and metamorphosis may be a component of the recruiting coralline alga itself, rather than of any microbial epibionts that are uncalcified. Ultrastructural immunohistochemical analyses, using antibodies against the purified morphogen, can now resolve this question unequivocally.

The hydrophobic behavior of the amphipathic morphogen can be exploited to test the role of larval chemosensory recognition in the control of substratum-specific settlement, metamorphosis, and recruitment in the natural environment. In steps toward that goal, our results demonstrate that the morphogen remains active after adsorption to beads of the t-butyl HIC resin; the HIC-resin-bound morphogen is not eluted from the beads by seawater and is equally active in the natural ocean environment and the laboratory. Attachment of the resin-bound inducer to surfaces coated with a silicone adhesive produces a potent artificial recruiting substratum corresponding to a morphogen-based chemical flypaper for *A. humilis* larvae. This substratum should prove useful for testing the relative contributions of chemosensory recognition of the algal morphogen and other factors to the control of settlement and metamorphosis under defined conditions in the laboratory, and to the control of recruitment and the maintenance of species isolation mechanisms in the natural environment. This is, to our knowledge, the first highly purified natural inducer of larval settlement and metamorphosis to be incorporated into an artificial substratum suitable for testing in the ocean. Advantages of this uniform, non-sticky, morphogen-based recruiting surface include avoidance of the complexity and variability of the natural algal substrata that usually confound the effects of chemical morphogen, textural complexity, cryptic animal predators, allelopathic competitors, and other chemical and biological interactions.

Use of such synthetic surfaces containing the natural morphogen should permit experimental resolution of the contributions of each of these factors.

Previous results demonstrated that the morphogen solubilized from *Hydrolithon boergesenii* is biologically specific, with respect to both the source of the molecule and the larvae capable of detecting or responding to it (Morse and Morse, 1991). Thus, two sympatric species of coralline algae that do not induce *A. humilis* larvae to metamorphose were shown not to contain the morphogen, and larvae of a sympatric coral that is not induced to settle or metamorphose by *H. boergesenii* also are not induced by the morphogen. However, larvae of the congeneric *A. tenuifolia* are induced to metamorphose by the intact alga and by the morphogen, suggesting that this cue may be group-specific (Morse and Morse, 1991). The morphogen-based chemical flypaper containing the purified inducer should be a useful tool for investigating the specificity of morphogen recognition by the cognate larval receptors and of the possibility that changes in this specificity serve as an axis for the evolution of niche-diversification and speciation.

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